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Symptom Attenuation by a Normally Virulent Satellite RNA of Turnip Crinkle Virus Is Associated with the Coat Protein Open Reading Frame

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Many satellite RNAs (sat-RNAs) can attenuate or intensify the symptoms produced by their helper virus. Sat-RNA C, associated with turnip crinkle virus (TCV), was previously found to intensify the symptoms of TCV on all plants in which TCV produced visible symptoms. However, when the coat protein open reading frame (ORF) of TCV was precisely exchanged with that of cardamine chlorotic fleck virus, sat-RNA C attenuated the moderate symptoms of the chimeric virus when Arabidopsis plants were coinoculated with the chimeric virus. Symptom attenuation was correlated with a reduction in viral RNA levels in inoculated and uninoculated leaves. In protoplasts, the presence of sat-RNA C resulted in a reduction of ~70% in the chimeric viral genomic RNA at 44 hr postinoculation, whereas the sat-RNA was consistently amplified to higher levels by the chimeric virus than by wild-type TCV. TCV with a deletion of the coat protein ORF also resulted in a similar increase in sat-RNA C levels in protoplasts, indicating that the TCV coat protein, or its ORF, downregulates the synthesis of sat-RNA C. These results suggest that the coat protein or its ORF is a viral determinant for symptom modulation by sat-RNA C, and symptom attenuation is at least partly due to inhibition of virus accumulation.

INTRODUCTION

Many plant RNA viruses are associated with dispensable, subviral RNAs known as satellite RNAs (sat-RNAs) that depend on the viral genomic RNA(s) for replication, encapsidation, and movement. Some sat-RNAs attenuate the symptoms of their helper virus, whereas a few exacerbate symptom expression (Roossinck et al., 1992). Because symptom attenuation by sat-RNAs is often accompanied by a reduced titer of the helper virus in plants, leading to a presumed reduction in the concentration of the elicitor of host pathogenesis, the sat-RNA is thought to outcompete the helper virus for replication factors. However, the virus titer is not always decreased, indicating that symptom attenuation by sat-RNAs may not involve a single mechanism (Harrison et al., 1987; Roossinck et al., 1992).

Symptom modulation properties of certain sat-RNAs must reside in the sequence or structure of the encapsidated (+) strand or (−) strand replicative intermediate, because the smaller sat-RNAs (194 to 400 bases), including those associated with turnip crinkle virus (TCV) and cucumber mosaic virus (CMV), do not encode detectable polypeptides in vivo (Roossinck et al., 1992). Specific nucleotide residues or regions of sat-RNAs associated with CMV (Masuta and Takanami, 1989; Devic et al., 1990; Jaegle et al., 1990; Sleat and Palukaitis, 1990; Wu and Kaper, 1992; Zhang et al., 1994) and peanut stunt virus (Naidu et al., 1992) have been implicated in symptom modulation. However, identification of such residues or regions has not furthered our understanding of how sat-RNAs modulate symptoms.

The identical sat-RNA can have a different effect on symptoms when associated with different helper viruses (Kaper et al., 1990; Sleat and Palukaitis, 1990; Sleat et al., 1994). For example, B2 and WL3 sat-RNAs induce chlorosis in tobacco when associated with subgroup II strains of CMV but attenuate symptoms when associated with subgroup I strains (Sleat and Palukaitis, 1990). These results indicate that the helper virus contributes to the symptom modulation properties of the associated sat-RNA. In addition, a single tobacco gene determines whether the CMV Y-sat-RNA produces yellow or green mosaic symptoms (Masuta et al., 1993), indicating a role for the host in sat-RNA–mediated symptom modulation.

Our understanding of how sat-RNAs interact with the helper virus and the host to intensify or ameliorate symptoms should benefit from the use of simple model systems. TCV is one of the smallest and simplest of the single-stranded, (+) sense RNA viruses and has a wide host range that includes Arabidopsis, a plant in increasing use as a model to study plant–pathogen interactions (Dangl, 1993). The TCV-M isolate contains a single genomic RNA of 4054 bases and is naturally associated with sat-RNA C (356 bases), a hybrid sat-RNA consisting of a nearly full-length avirulent sat-RNA (sat-RNA D) at the 5′ end joined to two fragments from the 3′ end of TCV

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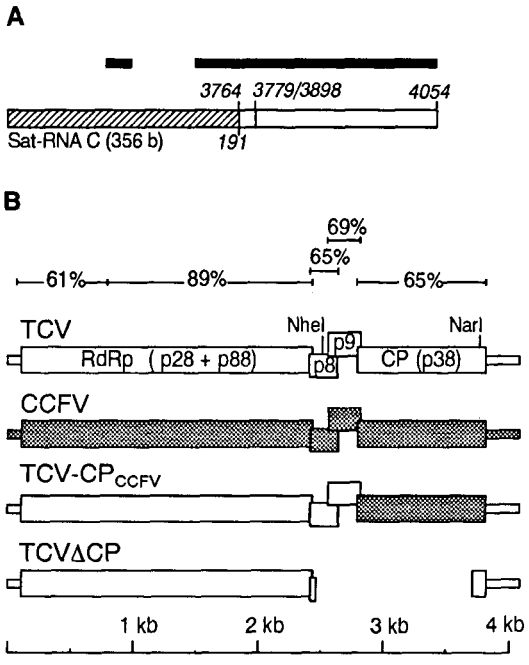

Figure 1. Schematic Representations of RNAs Used in This Study.

(A) Sat-RNA C. Sat-RNA C is composed of 191 bases of the 194-base TCV sat-RNA D at the 5′ end (hatched) joined to two regions of TCV genomic RNA at the 3′ end. Upper and lower numbers indicate corresponding positions from TCV genomic RNA and sat-RNA D, respectively. Black bars indicate regions involved in symptom intensification (Simon et al., 1988).

(B) Comparison of helper virus genomic RNAs. The percentage of amino acid sequence similarity between corresponding ORFs of TCV and CCFV is shown. Thick boxes represent ORFs, and thin bars represent untranslated regions. Shaded bars represent CCFV sequence, and open bars represent TCV sequence. The positions of restriction enzyme sites in the corresponding TCV cDNA used for the generation of TCVΔCP are shown. RdRp, RNA-dependent RNA polymerase.

genomic RNA (Figure 1A; Simon and Howell, 1986). Sat-RNA C has been shown to intensify symptoms on all hosts in which TCV produces visible symptoms (Li and Simon, 1990). The TCV-homologous region of sat-RNA C is involved in symptom intensification, along with a region between positions 79 and 100 (Simon et al., 1988). Transcripts synthesized in vitro from full-length cDNAs of TCV genomic RNA and sat-RNA C are biologically active (Simon and Howell, 1987; Oh et al., 1995).

With one exception, all Arabidopsis ecotypes inoculated with TCV genomic RNA alone display moderately severe stunting and bolt twisting, whereas inoculation with the genomic RNA and sat-RNA C results in severe systemic necrosis culminating in plant death within 19 days of inoculation (Li and Simon, 1990; Simon et al., 1992). The exception was ecotype Dijon (Di-0), which was nearly symptomless when inoculated with the genomic RNA with or without sat-RNA C (Simon et al., 1992). TCV genomic RNA is nearly always confined to the inoculated leaf of Di-0, while accumulating at a similar rate and to a similar level in protoplasts from susceptible and resistant ecotypes (Simon et al., 1992; Oh et al., 1995).

To identify viral determinants involved in the resistance, chimeric viruses were constructed between the genomic RNA of TCV-M and the related carmovirus, cardamine chlorotic fleck virus (CCFV). CCFV is able to infect systemically all ecotypes of Arabidopsis tested, including Di-0. Symptoms induced by CCFV are similar to but slightly less severe than TCV-induced symptoms on susceptible ecotypes. TCV, with its coat protein (CP) open reading frame (ORF) precisely replaced with the CCFV CP ORF (TCV-CP$_{CCFV}$), was able to infect Di-0 systemically, indicating that the CP, or its ORF, was involved in the resistance response (Oh et al., 1995).

In this report, we describe the unexpected consequence of coinoculating Arabidopsis ecotypes Di-0 and Columbia (Col-0) with TCV-CP$_{CCFV}$ and sat-RNA C. The normally virulent sat-RNA C attenuated the symptoms of the helper virus. Symptom attenuation was correlated with a decrease in viral genomic RNA levels in inoculated plants and protoplasts, along with an increase in accumulation of sat-RNA C in protoplasts. A similar increase in sat-RNA C levels was found in protoplasts when the TCV CP ORF was deleted, suggesting that the TCV CP, or its ORF, down-regulates the accumulation of sat-RNA C.

RESULTS

Sat-RNA C Ameliorates the Symptoms of TCV-CP$_{CCFV}$

As shown in Figure 1B, TCV encodes five polypeptides: p28 (function unknown), p88 (putative virus–encoded polymerase), p8 and p9 (required for cell-to-cell movement), and p38 (CP; also required for cell-to-cell movement) (Guilley et al., 1985; Carrington et al., 1987; Riviere and Rochon, 1990; Hacker et al., 1992). CCFV, recently discovered in the Australian Alps, has a similar genomic organization (Skotnicki et al., 1993). The protein products of TCV and CCFV share similarities ranging from 89% for the polymerase domain of p88 to 61% for that of p28. Unlike TCV, CCFV is not associated with detectable sat-RNAs or defective interfering (DI) RNAs (Oh et al., 1995).

Chimeric viruses were constructed using full-length, biologically active cDNAs of the TCV-M isolate and the Blue Lake isolate of CCFV by precisely exchanging the CP ORF using recombinant polymerase chain reaction methods (Oh et al., 1995). TCV with the CP ORF from CCFV was able to infect Arabidopsis ecotypes Col-0 and Di-0 systemically (Oh et al., 1995). To determine whether CCFV and TCV-CP$_{CCFV}$ could support TCV sat-RNA C and to determine the effect that the sat-RNA might have on symptom production, Arabidopsis ecotypes Col-0 and Di-0 were inoculated with TCV, CCFV, or TCV-CP$_{CCFV}$, with or without sat-RNA C. Symptoms were assessed visually at various times up to 19 days postinoculation (dpi). As shown in Figure 2, CCFV, with or without sat-RNA C, had stunted and twisted bolts. In addition, young rosette leaves contained chlorotic veins that were visible after 6 dpi (data not shown). Col-0 plants inoculated with TCV alone developed slightly more severe symptoms than did those Col-0

plants inoculated with CCFV. The symptoms consisted of severe stunting, curling of leaves and bolts, and progressive necrosis of rosette leaves starting from the tip. Inoculation of Col-0 plants with TCV and sat-RNA C resulted in inhibition of bolting and a spreading necrosis that covered the whole plant by 19 dpi (Figure 2A). No symptoms were discernible on most Di-0 plants inoculated with TCV, with or without sat-RNA C (Figure 2B), with the exception of a hypersensitive reaction on the inoculated leaves that has been described previously (Simon et al., 1992).

Figure 2. Effect of Sat-RNA C on Symptom Expression of TCV, CCFV, and TCV-CP_{CCFV}.

(A) Using ecotype Col-0, sat-RNA C intensifies the symptoms of TCV, attenuates symptoms of TCV-CP_{CCFV} (T/C), and has no effect on CCFV. **(B)** Using ecotype Di-0, sat-RNA C has no effect on plants inoculated with TCV and CCFV but attenuates symptoms of TCV-CP_{CCFV} (T/C). Plants were inoculated with viral RNA(s) or inoculation buffer (Mock) as indicated on the two oldest leaves of seedlings at the four- or five-leaf stage and were photographed at 19 dpi. +sat-C indicates that sat-RNA C was included in the inoculum.

TCV-CP_{CCFV} alone produced moderate symptoms similar to those produced by CCFV on both Col-0 and Di-0 plants (Figure 2), except that the symptoms in young rosette leaves became visible after 8 dpi (data not shown). Surprisingly, all Col-0 and Di-0 plants inoculated with TCV-CP_{CCFV} and sat-RNA C were symptomless (Figure 2), with the exception of a few plants that had a mild mosaic pattern on young rosette leaves (data not shown). This result indicated that instead of exhibiting its normal property of symptom intensification, sat-RNA C was ameliorating the symptoms of TCV-CP_{CCFV}. Furthermore, because TCV and TCV-CP_{CCFV} differed only in the CP ORF, the CP or its ORF is a viral determinant for symptom modulation by sat-RNA C in Arabidopsis plants.

Sat-RNA C Limits the Accumulation of TCV-CP_{CCFV} in Inoculated and Uninoculated Leaves of Col-0 and Di-0 Plants

The lack of symptoms exhibited by Arabidopsis when inoculated with TCV-CP_{CCFV} and sat-RNA C could be due to a sat-RNA–mediated reduction in the amount of virus in the infected plant or an induced tolerance of the plant to virus infection. To determine whether sat-RNA C was affecting the level of viral RNA accumulating in Col-0 and Di-0 plants, seedlings at the four- or five-leaf stage were inoculated with CCFV, TCV, or TCV-CP_{CCFV}, with or without sat-RNA C, on the oldest leaf pair. At 10 dpi, inoculated and uninoculated leaves were collected, and total RNA was isolated and subjected to RNA gel blot analysis using probes specific for viral genomic RNA or sat-RNA C. As shown in Figure 3A, CCFV, TCV, and TCV-CP_{CCFV} accumulated to comparable levels in inoculated leaves of Col-0 plants in the absence of sat-RNA C (for simplicity, references to the names of the viruses indicate the viral genomic RNAs). The addition of sat-RNA C resulted in a substantial reduction in the amount of TCV-CP_{CCFV} but did not consistently affect the level of CCFV or TCV. Sat-RNA C accumulated to levels fourfold higher when associated with TCV than with TCV-CP_{CCFV} and was not detected in leaves inoculated with CCFV. In uninoculated Col-0 leaves in the absence of sat-RNA C, TCV accumulated to a higher level at 10 dpi than did CCFV and TCV-CP_{CCFV}. The addition of sat-RNA C to the inocula did not substantially affect the levels of CCFV and TCV, while significantly reducing the spread of TCV-CP_{CCFV}. High levels of sat-RNA C were found in uninoculated leaves only in the presence of TCV.

In Di-0 plants (Figure 3B), TCV, either with or without sat-RNA C, accumulated to low levels in inoculated leaves and was undetectable in uninoculated leaves, as previously reported (Simon et al., 1992; Oh et al., 1995). CCFV accumulated in Di-0 plants to levels similar to those found in CCFV-infected Col-0 plants and did not support the accumulation of sat-RNA C to detectable levels. TCV-CP_{CCFV} accumulated to similar levels in inoculated and uninoculated leaves of Di-0 and Col-0 plants in the absence of sat-RNA C. The addition of sat-RNA C substantially reduced the levels of

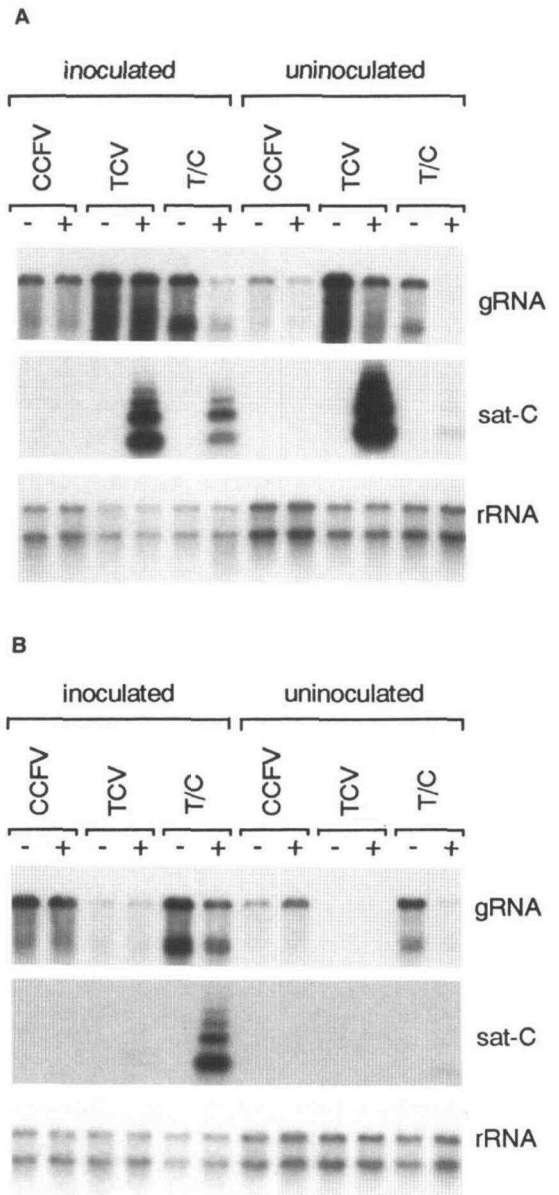

Figure 3. Effect of Sat-RNA C on Accumulation of Viral Genomic RNA in Arabidopsis Plants.

(A) Ecotype Col-0.

(B) Ecotype Di-0.

The two oldest leaves of 40 seedlings at the four- or five-leaf stage were inoculated with viral genomic RNA with (+) and without (−) sat-RNA C. Total RNA was isolated from inoculated and uninoculated leaves collected at 10 dpi. Three micrograms of total cellular RNA per lane was denatured and loaded onto 1.2% nondenaturing agarose gels. RNA gel blots were hybridized sequentially with probes specific for viral genomic RNAs (gRNA), sat-RNA C (sat-C), and ribosomal RNAs (rRNA). The genomic RNA probes hybridized to both genomic (the slowest migrating species) and subgenomic viral RNAs. As with many sat-RNAs, sat-RNA C is found in monomeric and multimeric forms. T/C, TCV-CP_{CCFV}.

TCV-CP_{CCFV} in uninoculated leaves but had a lesser effect in inoculated leaves. These results suggest that (1) TCV-CP_{CCFV}, but not CCFV, can support sat-RNA C, and (2) sat-RNA C substantially reduces the accumulation of TCV-CP_{CCFV} in inoculated and uninoculated leaves of Col-0 and Di-0 plants.

The effect of sat-RNA C on the distribution of viral genomic RNA in individual plants was assayed using whole-plant in situ hybridizations (Simon et al., 1992; Oh et al., 1995). Col-0 and Di-0 seedlings at the four- or five-leaf stage were inoculated with TCV or TCV-CP_{CCFV}, with or without sat-RNA C, on one of their two oldest leaves, and plants were collected at different times postinoculation. As depicted in Figure 4, TCV, when inoculated alone, was detected in the inoculated leaf of Col-0 by 2 dpi and in uninoculated leaves by 3 dpi, which agrees with previous results (Oh et al., 1995). The addition of sat-RNA C to the inoculum did not affect the rate of virus spread but did reproducibly cause the virus to become more concentrated in the youngest leaves by 10 dpi (Figure 4 and data not shown). In Di-0 plants, TCV was limited to low levels within the inoculated leaves of most plants, regardless of the presence of sat-RNA C. As described previously (Simon et al., 1992), Di-0 plants occasionally display mild symptoms, and in this experiment, one plant inoculated with TCV and sat-RNA C showed evidence of a systemic infection at 10 dpi (Figure 4).

As with TCV, TCV-CP_{CCFV} was first detected in the inoculated leaf at 2 dpi. However, long-distance movement into uninoculated leaves was delayed when compared with TCV, requiring an additional 4 to 6 days; just 1 day was required for TCV (Figure 4). Inclusion of sat-RNA C in the inoculum containing TCV-CP_{CCFV} resulted in reduced amounts of genomic RNA in the inoculated leaf and a lack of detectable virus in uninoculated leaves by 10 dpi. This result confirms that sat-RNA C reduces the level of TCV-CP_{CCFV} in inoculated and uninoculated leaves of Col-0 and Di-0 plants.

Sat-RNA C Inhibits the Accumulation of TCV-CP_{CCFV} in Protoplasts

The decrease in TCV-CP_{CCFV} levels in the plant in the presence of sat-RNA C could be due to a sat-RNA–mediated effect on either virus replication/stability or virus movement. If sat-RNA C is affecting the replication/stability of the genomic RNA, then this property should be discernible in Arabidopsis protoplasts. Col-0 protoplasts were prepared from seedling callus cultures and inoculated with TCV or TCV-CP_{CCFV}, with or without sat-RNA C. Total RNA was isolated from protoplasts collected between 0 and 44 hr postinoculation and subjected to RNA gel blot analysis. The results of one of four replicate experiments are presented in Figure 5A, and the normalized densitometric scanning data for the four experiments are presented in Figure 5B. In the absence of sat-RNA C, TCV and TCV-CP_{CCFV} accumulated at similar rates and to similar levels. The addition of sat-RNA C reduced the level of TCV by ~30% and the level of TCV-CP_{CCFV} by ~70% at 44 hr postinoculation. Sat-RNA C also consistently accumulated to higher levels in the presence of TCV-CP_{CCFV} rather than TCV. These results, together with the results in the plants, suggest that (1) sat-RNA C levels are elevated in the presence of TCV-CP_{CCFV} compared with wild-type TCV, and (2) the amount of TCV-CP_{CCFV}, when compared with TCV, is more substantially reduced by the presence of sat-RNA C.

Sat-RNA C Accumulation in Protoplasts Is Inhibited by TCV CP or Its ORF

The increase in sat-RNA C levels when associated with TCV-CP_{CCFV} could be due to the presence of the CCFV CP (or its ORF) or the absence of the TCV CP (or its ORF). If the TCV CP down-regulates the accumulation of sat-RNA C, then TCV with a deletion of the CP ORF should support sat-RNA C to a higher level when compared with wild-type TCV. Hacker et al. (1992) reported that the TCV-B isolate with a deletion encompassing ORFs encoding p9, most of p8, and most of the CP was still able to replicate in *Brassica campestris* protoplasts, although the deleted virus was unable to infect whole plants systemically. For this study, the identical deletion was generated in TCV-M (TCVΔCP; Figure 1B), and transcripts of TCVΔCP, TCV-CP_{CCFV}, and TCV were used to inoculate Col-0 protoplasts, with or without sat-RNA C. As shown in Figure 6, TCVΔCP was able to accumulate in protoplasts. However, a substantial amount of RNA hybridizing to the probe migrated faster than the full-length TCVΔCP RNA, suggesting that in the absence of the CP, the genomic RNA was more susceptible to degradation. Although the level of full-length TCVΔCP in the presence of sat-RNA C was 13-fold less than the level of TCV with sat-RNA C at 44 hr postinoculation (as determined from normalized scanning of autoradiograms from two replicate experiments), sat-RNA C levels were an average of threefold higher using TCVΔCP, as opposed to wild-type TCV, as the helper virus. The level of sat-RNA C in the presence of TCVΔCP was similar to the level reached using TCV-CP_{CCFV} as the helper (an average of a fourfold increase over wild-type TCV for these two experiments), suggesting that the TCV CP (or its ORF) is reducing the replication/stability of sat-RNA C.

DISCUSSION

We demonstrated that replacement of the CP ORF in a helper virus genome can convert a virulent sat-RNA into an ameliorative sat-RNA. In the presence of sat-RNA C, the level of TCV-CP_{CCFV}, but not TCV, was substantially reduced throughout Col-0 plants and in uninoculated leaves of Di-0, as assayed by RNA gel blot analysis and whole-plant in situ hybridizations. This substantial reduction in virus levels is most likely responsible for the lack of symptoms outside the inoculated leaves.

We believe that the symptom attenuation in plants and reduction in viral RNA levels in protoplasts were due to the presence

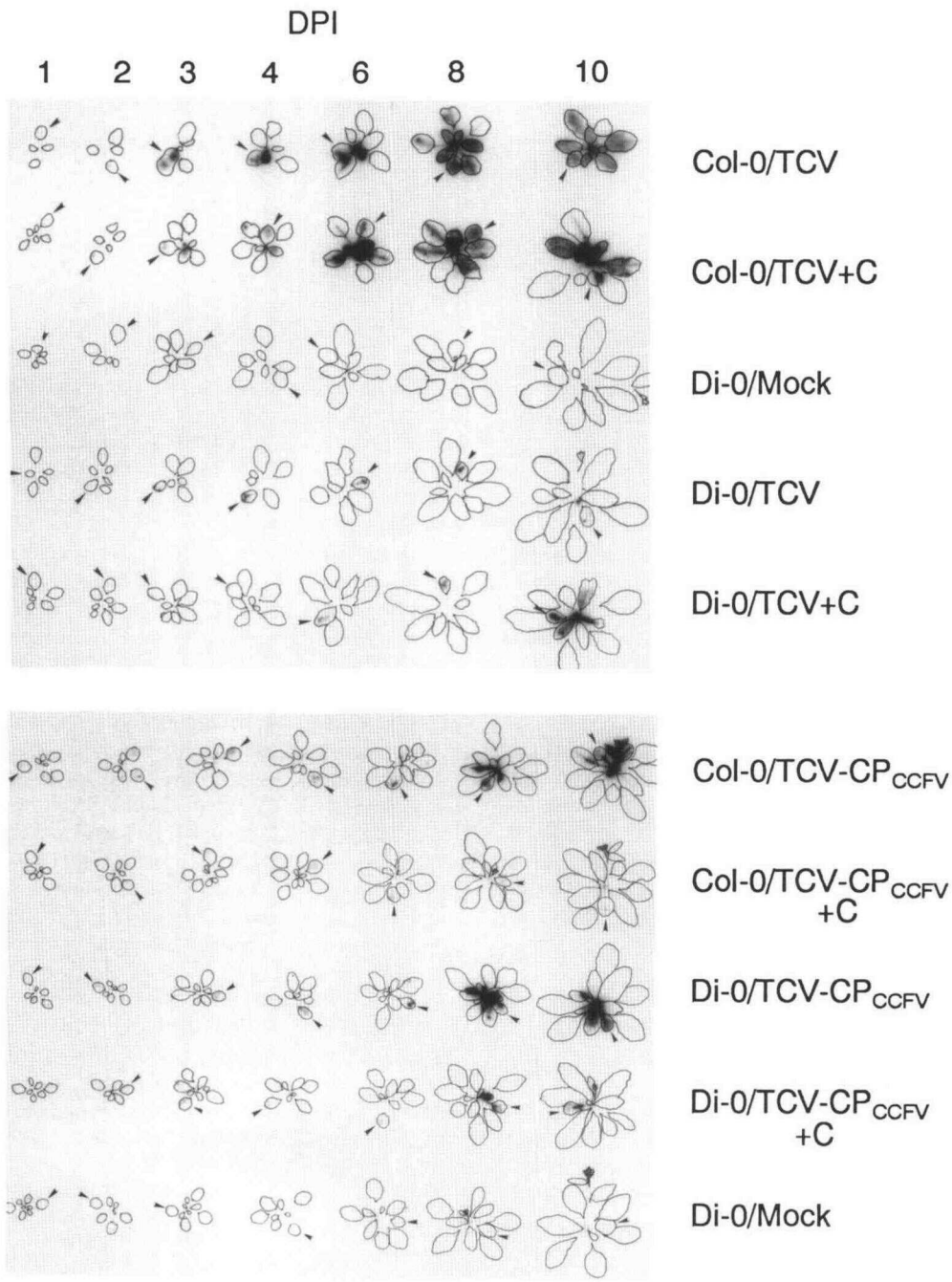

Figure 4. Effect of Sat-RNA C on Accumulation of TCV and TCV-CP$_{CCFV}$ in Arabidopsis Plants as Detected by Whole-Plant in Situ Hybridization.

Two of the oldest leaves of Col-0 and Di-0 seedlings at the four- or five-leaf stage were inoculated with TCV or TCV-CP$_{CCFV}$, with (+C) or without sat-RNA C. Plants were collected on the days postinoculation (dpi), as indicated, and probed with a ^{32}P-labeled oligonucleotide specific for TCV and TCV-CP$_{CCFV}$, as described in Methods. Outlines of leaves were traced on a clear plastic sheet and photographed with the autoradiogram. Arrows point to the inoculated leaves. Mock-inoculated plants (Mock) received the inoculation buffer alone.

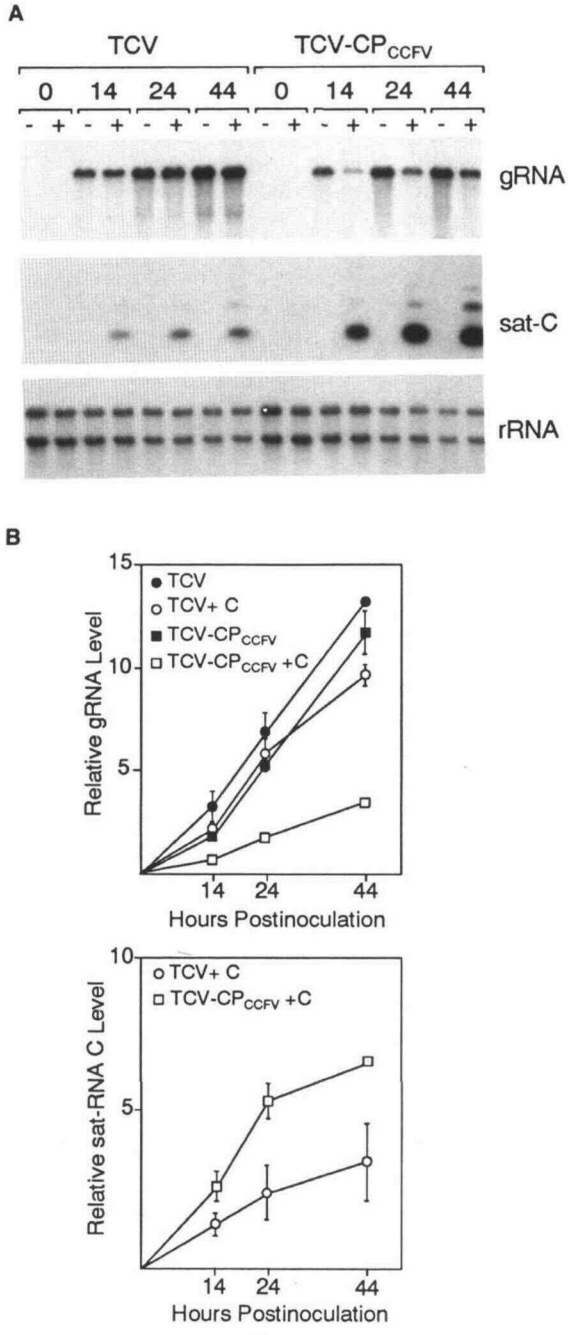

Figure 5. Accumulation of TCV, TCV-CP$_{CCFV}$, and Sat-RNA C in Protoplasts.

(A) Levels of TCV and TCV-CP$_{CCFV}$ reduced in the presence of sat-RNA C. Col-0 protoplasts were inoculated with full-length transcripts of TCV or TCV-CP$_{CCFV}$, with (+) or without (−) sat-RNA C as indicated. Total RNA, isolated from samples collected at the times postinoculation (in hours) indicated above the lanes, was subjected to RNA gel blot analysis. The blot was hybridized sequentially with probes specific for genomic TCV and TCV-CP$_{CCFV}$ RNAs (gRNA), sat-RNA C (sat-C), and ribosomal RNAs (rRNA).

(B) Normalized results of replicate experiments. Autoradiograms from four replicate experiments as illustrated in **(A)** were scanned with a densitometer, and viral genomic and sat-RNA levels were normalized to the levels of rRNAs. Error bars denote standard error. Top, viral genomic RNA (gRNA) levels; bottom, sat-RNA C levels. +C indicates that sat-RNA C was included in the inoculum.

of sat-RNA C and not due to possible de novo generation of DI RNAs (subviral RNAs derived from the viral genomic RNA). Although DI RNAs can be associated with symptom attenuation and reduction in viral genomic RNA levels (Hillman et al., 1987), the one TCV DI RNA that has been examined was virulent, producing symptoms similar to those normally associated with sat-RNA C (Li et al., 1989). In addition, no unexpected subviral RNA species were visible in the ethidium bromide–stained gels subjected to RNA gel blot analysis in Figures 3, 5, and 6 (data not shown). Although the RNA gel blot autoradiogram in Figure 6 (which was overexposed to reveal the low levels of TCV-CP$_{CCFV}$ and TCVΔCP in the presence of sat-RNA C) indicates the presence of RNA species migrating faster than the genomic RNA, these reproducible species were present in protoplasts inoculated with or without sat-RNA C. The levels of these species were proportional to the level of viral genomic RNA, suggesting that they represent degradation products.

In Col-0 protoplasts, the presence of sat-RNA C was associated with an average decrease of ~70% in the amount of TCV-CP$_{CCFV}$ at 44 hr postinoculation, compared with a decrease of only ~30% for TCV. In addition, sat-RNA C levels were on average two- to fourfold higher in the absence of the TCV CP ORF (TCV-CP$_{CCFV}$ or TCVΔCP). This reproducible increase in sat-RNA C levels using TCV-CP$_{CCFV}$ as the helper virus in Col-0 protoplasts was not evident in inoculated leaves of Col-0 (Figure 3A). However, sat-RNA accumulation in leaves requires both movement and replication, which are dependent on the level of the helper virus. The amount of TCV-CP$_{CCFV}$ was much lower than that of TCV in inoculated Col-0 leaves in the presence of the sat-RNA, which could account for the lower level of sat-RNA C associated with TCV-CP$_{CCFV}$. The substantial decrease in TCV-CP$_{CCFV}$ in protoplasts in the presence of sat-RNA C suggests that sat-RNA C ameliorates the symptoms of TCV-CP$_{CCFV}$ by better competing for replication factors in the absence of the TCV CP ORF, thereby reducing the level of helper virus below a threshold required for systemic infection. A decrease in TCVΔCP levels was also found in protoplasts in the presence of sat-RNA C (see Figure 6). However, because TCV requires the CP for movement in plants, we are unable to test whether sat-RNA C is able to affect the symptoms of TCVΔCP.

Sat-RNA symptom modulation most likely involves a complex interaction among sat-RNA, helper virus, and host plant (Waterworth et al., 1979; Kaper, 1992; Masuta et al., 1993). To date, much work has focused on elucidating sat-RNA

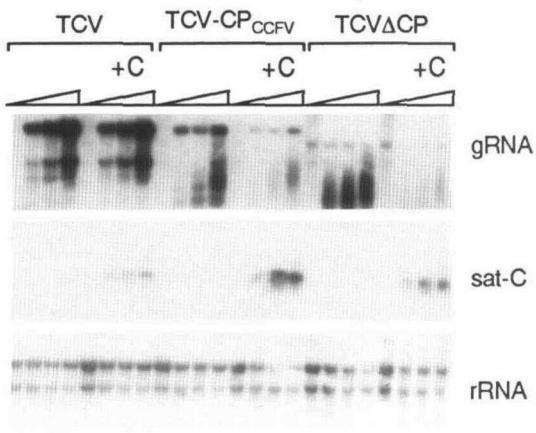

Figure 6. Inhibition of Sat-RNA C Accumulation by TCV CP ORF in Col-0 Protoplasts.

Protoplasts were inoculated with TCV, TCV-CP$_{CCFV}$, or TCVΔCP with (+C) or without sat-RNA C. Samples were collected at 0, 14, 24, and 44 hr postinoculation (depicted as triangles increasing from left to right above the lanes), and total RNAs isolated from these samples were analyzed as described in Figure 5A. The autoradiogram was overexposed to detect the low levels of TCVΔCP in the presence of sat-RNA C. The slowest migrating species hybridizing to the genomic RNA probe are at the position of full-length genomic RNA.

sequences that, when altered, change the sat-RNA's symptom modulation properties (Roossinck et al., 1992; Wu and Kaper, 1992; Zhang et al., 1994). The role of the helper virus is much less defined. WLM2 sat-RNA induces necrosis in tomato when associated with subgroup II strains of CMV, whereas symptoms are ameliorated when associated with subgroup I strains of CMV (Sleat et al., 1994). The viral determinant involved in the differential symptom expression was broadly mapped to RNA 2, which encodes the polymerase subunit of the RNA-dependent RNA polymerase. In turnip, mutations in the RNA-dependent RNA polymerase ORF of TCV were found to affect symptom modulation by sat-RNA C (Collmer et al., 1992; Oh et al., 1995). In the study by Collmer et al. (1992), the authors examined why sat-RNA C was less virulent when associated with the TCV-B isolate than with the TCV-JI isolate. They correlated increased virulence with an increased rate of sat-RNA C accumulation at early times after inoculation of protoplasts in the presence of the TCV-JI isolate.

In our study, exchanging the TCV CP ORF with the corresponding ORF from CCFV altered the symptom modulation properties of sat-RNA C from virulent to ameliorative, indicating that the viral CP (or its ORF) can also be a determinant of sat-RNA symptom modulation. Interestingly, symptom amelioration was associated with an increased rate of sat-RNA accumulation in protoplasts, in direct contrast with the Collmer et al. (1992) report. In addition to our finding that the viral CP (or its ORF) is involved in sat-RNA–mediated symptom modulation, the CP has been implicated in symptom production by the helper virus. Single amino acid alterations in the CP of CMV (Shintaku et al., 1992) or alfalfa mosaic virus (Neeleman et al., 1991) induced chlorosis in tobacco or changed symptoms from mildly chlorotic to severely necrotic, respectively. In studies using TCV-B, the TCV CP was hypothesized to contain pathogenesis determinants, because several amino acid alterations resulted in milder or more severe symptoms than normal on *Nicotiana benthamiana* in the absence of any sat-RNAs (Heaton et al., 1991). Determinants for resistance have also been found associated with CPs; a reduction in subunit interactions was recently hypothesized to expose a site on the CP that interacts with the *N'* gene product of tobacco, resulting in hypersensitive resistance to tobacco mosaic virus (Culver et al., 1994). In these studies, the CP was thought to play an active role in symptom production, either by encapsidating and thereby stabilizing viral RNAs or by having multiple distinct determinants that can be exposed (or hidden) by changes in the protein structure.

In our study, lack of the TCV CP ORF was correlated with a two- to fourfold increase in sat-RNA C levels in protoplasts, suggesting that the TCV CP (or its ORF) is down-regulating the accumulation of sat-RNA C. Although the TCV CP is not required for the accumulation of viral genomic RNA in protoplasts, considerable degradation of the viral genomic RNA was found in the absence of the CP (Figure 6). CPs have also been shown to affect the synthesis or stability of other viral genomic RNAs (Sacher and Ahlquist, 1989; Allison et al., 1990; Ishikawa et al., 1991; Marsh et al., 1991; van der Kuyl et al., 1991; Chapman et al., 1992). How the TCV CP (or its ORF) reduces the accumulation of sat-RNA C in protoplasts is not clear. The TCV CP may restrict the synthesis of sat-RNA C by binding to the RNA and masking hairpins required for sat-RNA C transcription (C. Song and A.E. Simon, unpublished data). Because extracts from TCV-infected plants are able to transcribe complementary strands of sat-RNA C using (+) or (−) strand template (Song and Simon, 1994), it should be possible to test whether the TCV CP affects the replication of sat-RNA C in vitro.

The question remains whether the ~70% sat-RNA C–mediated decrease in the accumulation of TCV-CP$_{CCFV}$ found in protoplasts is sufficient to account for the nearly undetectable levels of virus in uninoculated leaves of Col-0 and Di-0. Sat-RNA C also reduced the amount of TCV in protoplasts by ~30%; however, in ecotypes of Arabidopsis other than Di-0, symptoms are intensified, not lessened. Because the amount of sat-RNA C is directly proportional to the level of symptom intensification in turnip (A.E. Simon, unpublished data), it is difficult to hypothesize how an increase in sat-RNA C levels might contribute to symptom amelioration. However, the possibility cannot be ruled out that high initial rates of sat-RNA C accumulation may induce host defense responses, leading to a restriction of virus movement. The slower spread of TCV-CP$_{CCFV}$ through Arabidopsis, compared with TCV or CCFV (Figure 4), may also be a factor in restriction of systemic virus accumulation. We are currently testing additional TCV-associated sat-RNAs and DI RNAs, both virulent and avirulent, for their effects on the biological activity of TCV-CP$_{CCFV}$.

METHODS

Origin of Helper Viruses and Inoculation of Plants

Cloned cDNAs for the severe isolation of turnip crinkle virus TCV-M (TCVms) and the Blue Lake isolate of cardamine chlorotic fleck (CCFV; CCFV-BL) were used in this study (Oh et al., 1995). The construction of TCV-CP_{CCFV} has been described by Oh et al. (1995). TCV with a deletion of the coat protein (CP) open reading frame (ORF) (TCVΔCP) was constructed by deleting the region between the NheI site (position 2458) and the NarI site (position 3626) from the full-length cDNA of TCVms in plasmid pT7TCVms (Oh et al., 1995). Plants (*Arabidopsis thaliana* ecotypes Columbia [Col-0] and Dijon [Di-0]) were grown in growth chambers at 20°C as described by Li and Simon (1990). Plant seedlings were mechanically inoculated with 0.1 mg/mL total RNA isolated from Arabidopsis plants previously inoculated with full-length transcripts synthesized from cloned cDNAs corresponding to the genomic RNAs of TCVms, TCV-CP_{CCFV}, or CCFV-BL (Oh et al., 1995). Total RNA was extracted from Arabidopsis plants as previously reported (Simon et al., 1992). For coinoculation experiments, 0.01 mg/mL of full-length transcripts synthesized from the cloned cDNA of satellite RNA C (sat-RNA C; Song and Simon, 1994) was included in the inoculum. All transcripts were synthesized using T7 RNA polymerase (Simon et al., 1992).

RNA Gel Blot Analysis

Total RNA isolated from Arabidopsis plants or protoplasts (Simon et al., 1992) was denatured by heating in 50 to 75% formamide and then subjected to electrophoresis through nondenaturing 1.2% agarose gels. Gels were then rinsed with H_2O, incubated for 1 hr in 6% formaldehyde, and soaked in 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 30 min before transfer to NitroPlus membranes (Micron Separations Inc., Westboro, MA). Hybridization and washings were performed as described previously (Simon et al., 1992). Viral RNA and sat-RNA probes were oligonucleotides labeled with γ-^{32}P-ATP by using T4 polynucleotide kinase. An oligonucleotide complementary to positions 958 to 978 of TCVms and positions 952 to 972 of CCFV-BL was used for experiments probing viral genomic RNA accumulating in protoplasts. The viral genomic RNA probe for blots of total RNA from infected plants was an oligonucleotide complementary to positions 3892 to 3912 of TCVms and positions 3914 to 3934 of CCFV-BL; this oligonucleotide also hybridizes to viral subgenomic RNAs (1.7 and 1.45 kb for TCV). The sat-RNA C–specific probe was an oligonucleotide complementary to positions 175 to 199 of sat-RNA C. The rRNA probe has been described previously (Simon et al., 1992). Autoradiograms were scanned with a densitometer, and values were normalized to the level of rRNA.

Whole-Plant in Situ Hybridization

The protocol for whole-plant in situ hybridization has been described elsewhere (Oh et al., 1995). Briefly, seedlings were inoculated with TCV or TCV-CP_{CCFV}, with and without sat-RNA C, on one of their two oldest leaves. Plants, collected at various days postinoculation (dpi), were treated in succession with 95% ethanol; 0.1 mM NaN_3, 0.1% SDS, 10 mM EDTA, pH 8.0, 0.5 mg/mL self-digested Pronase (Sigma); 0.2 N HCl; and 2 × SSC. After drying, plants were subjected to prehybridization, hybridization, and washing as previously described (Oh et al., 1995). After autoradiography, the outlines of leaves were traced onto clear plastic, which was then photographed together with the autoradiogram.

Preparation and Inoculation of Protoplasts

Col-0 protoplasts were prepared and inoculated as described previously (Guzman and Ecker, 1988; Simon et al., 1992). Eighty micrograms of full-length transcripts from cloned cDNAs of TCVms, TCV-CP_{CCFV}, or TCVΔCP was used to inoculate 2 × 10^7 protoplasts in the presence or absence of 8 μg of sat-RNA C transcripts.

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